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Research article

PnSAG1, an E3 ubiquitin ligase of the Antarctic moss *Pohlia nutans*, enhanced sensitivity to salt stress and ABA

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ABSTRACT

Plant U-box (PUB) E3 ubiquitin ligases play crucial roles in the plant response to abiotic stress and the phytohormone abscisic acid (ABA) signaling, but little is known about them in bryophytes. Here, a representative U-box armadillo repeat (PUB-ARM) ubiquitin E3 ligase from Antarctic moss *Pohlia nutans* (PnSAG1), was explored for its role in abiotic stress response in *Arabidopsis thaliana* and *Physcomitrella patens*. The expression of PnSAG1 was rapidly induced by exogenous abscisic acid (ABA), salt, cold and drought stresses. PnSAG1 was localized to the cytoplasm and showed E3 ubiquitin ligase activity by in *vitro* ubiquitination assay. The *PnSAG1*-overexpressing *Arabidopsis* enhanced the sensitivity with respect to ABA and salt stress during seed germination and early root growth. Similarly, heterogeneous overexpression of PnSAG1 in *P. patens* was more sensitive to the salinity and ABA in their gametophyte growth. The analysis by RT-qPCR revealed that the expression of salt stress/ABA-related genes were downregulated in PnSAG1-overexpressing plants after salt treatment. Taken together, our results indicated that PnSAG1 plays a negative role in plant response to ABA and salt stress.

1. Introduction

Higher plants are frequently subjected to adverse environmental conditions, owing to either biotic factors (e.g., pathogens and herbivores) or abiotic factors (e.g., extreme temperature, water availability and high salinity), which severely affect plant growth and development. To survive under such (ever-changing environment) severe environmental stresses, sessile plants have evolved complicated protective strategies that involve integrated molecular, cellular, and metabolic programs (Hirayama and Shinozaki, 2010; Hummel et al., 2010). Stress signals are transduced to activate stress response genes and alter metabolic rates and ion channel permeability (Hasegawa et al., 2000). Among these strategies, the ubiquitin (Ub)-26S proteasome system, which mediates post-translational modification of proteins or degradation of the target protein, is one of the most prominent mechanisms during plants response to developmental cues or adaption to various environmental stresses (Smalle and Vierstra, 2004; Santner and Estelle, 2010; Stone, 2019).

E3 ubiquitin ligase, which delivers ubiquitin from the E2 (ubiquitin-conjugating enzyme) ubiquitin intermediate to the target protein, is the key factor in the ubiquitination system that determines the specificity of the extensive range of substrate (Vierstra, 2009; Sadanandom et al., 2012). Plants have larger numbers of different E3 ligases than other eukaryotes, with over 1400 different E3s in *Arabidopsis thaliana* genome and over 1300 members of E3 ligases in the rice genome (Stone et al., 2005; Du et al., 2009; Vierstra, 2009). According to the mechanism of action and the presence of special domains, E3 ligases can be largely categorized into four families: the E6-AP Carboxyl Terminus (HECT), Really Interesting New Gene (RING), Cullin (Cul)-RING ligases (CRLs) and U-box ligases (Smalle and Vierstra, 2004; Stone and Callis, 2007; Yee and Goring, 2009). Among them, the U-box domain contains 70 conserved amino acids and forms a tertiary scaffold structure resembling that of the (a modified) RING-finger domain (Azevedo et al., 2001; Ohi et al., 2003). Compared with the 2 U-box genes in *Saccharomyces cerevisiae* and 21 in human, there are 64, 77 and 125 U-box genes have been annotated in *Arabidopsis,* the rice and soybean genome, respectively, indicating that their function in diverse biological processes (Wiborg et al., 2008; Zeng et al., 2008; Wang et al., 2016).

U-box E3s from monocots and dicotyledons are involved in the growth and developmental processes of plants (Kinoshita et al., 2015; Somssich et al., 2016; Liu et al., 2018), plant innate immunity against pathogens (Ishikawa et al., 2014; Liu et al., 2015), and hormone regulation (Antignani et al., 2015; Jung et al., 2015; Zhou et al., 2018). In addition, a growing number of U-box E3s are also found to play roles in

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regulating plants responses to abiotic stress. For example, the *Arabidopsis* PUB22 and PUB23 negatively regulate drought tolerance by facilitating pyrabactin resistance-like 9 (PYL9) degradation only when they interact with ABA receptors (Zhao et al., 2017). Rice U-box E3 ligases *OsPUB2*- and *OsPUB3*-overexpressing plants markedly enhanced cold tolerance in terms of survival rates, chlorophyll content, ion leakage, and the expression levels of cold stress-inducible marker genes, suggesting that these genes may be highly valuable for improving crops resistance (Byun et al., 2017). The *Arabidopsis* PUB30 is a negative factor of salt stress response in seed germination through specifically ubiquitinating salt-tolerant gene BRI1 kinase inhibitor 1 (BKI1) and brassinosteroids signaling (Hwang et al., 2015; Zhang et al., 2017c). Overexpression of *Glycine max GmPUB8* displays hypersensitivity to osmotic and salt stresses during seed germination and seedling growth, and inhibits ABA- and mannitol-mediated stomatal closure with less induction of drought stress-related genes (Wang et al., 2016). *Triticum aestivum TaPUB1* improve germination and seedling survival rates, photosynthetic rate, water retention, as well as reduce the accumulation of reactive oxygen species (ROS) under drought stress, suggesting this gene positively modulates plant drought stress resistance potential by improving their antioxidant capacity (Zhang et al., 2017a).

Mosses are the most visually recognized organisms on extensive ice-free areas of Antarctica, and they are capable of growing and thriving under the physiologically extreme conditions on Earth (Pisa et al., 2013). Thus, the Antarctic mosses must have physiological and genetic characteristics that make them highly adapt to the harsh environment (Royles and Griffiths, 2015). For example, UV-B-absorbing compounds (UVAC) including red pigments within the cell walls have high photoprotection for Antarctic mosses surviving in multiple stresses (desiccation, naturally high UV and visible light) (Waterman et al., 2018). Overexpression of PaFKBP12, a peptidyl prolyl isomerase gene from the polar moss *Polytrichastrum alpinum*, resulted in more tolerance to heat, ABA, salt and drought stresses during root elongation and shoot growth in *Arabidopsis* (Alavilli et al., 2018). Several receptor like kinases, jasmonate ZIM-domain protein and genes of flavonoid synthesis pathways are involed in the adaptation of Antarctic moss *Pohlia nutans* to the polar environment (Wang et al., 2017a; Liu et al., 2019; Yao et al., 2019). In this study, we isolate a PUB-ARM ubiquitin E3 ligase gene from the Antarctic moss *Pohlia nutans*, which overexpression rendered bryophyte *physcomitrella patens* and *Arabidopsis* plants hypersensitive to ABA and salt stress (PnSAG1). These results provide new insights into the functions of PUB proteins in Antarctica moss.

2. Materials and methods

2.1. Plant materials and stress treatments

Pohlia nutans was obtained from the vicinity of the Great Wall Station in Antarctica in March 2014. *P. nutans* was cultured in the pots soil containing Base Substrate (Klasmann-Deilmann, Geeste, Germany) and local soil (1:1) and incubated at 16 °C with 70% relative humidity and 70 μmol m^{-2}·s^{-1} light. The mosses were treated with abiotic stresses including salinity (200 mM), dehydration (20% (w/v) polyethylene glycol 6000 solution), cold (4 °C) and abscisic acid (ABA, 50 μM) for the indicated times. The aerial portions of the shoots were harvested and frozen immediately in liquid nitrogen after different stresses.

Arabidopsis thaliana used for wild type (WT) and transformation were grown on sterile soil and kept in the greenhouse at 22 °C with 60% relative humidity and light intensity of 80 μmol m^{-2}·s^{-1}. After growing 4 weeks with 8 h light, they were transferred to 16 h light greenhouse for the plants transformation and maturation.

Physcomitrella patens were grown on BCD medium at 25 °C with 60 μmol m^{-2}·s^{-1} light. After 3 weeks with 16 h light, gametophyte tissues were sufficiently ground by a tissue homogenizer and then evenly distributed on cellophane overlaid BCD medium supplemental with 5 mM diammonium (+) tartrate to form protonema. The new protonema was used for the plants transformation.

2.2. Gene cloning and bioinformatics analysis

The HMMER program was used to search the ubiquitin ligases from the Antarctic moss *P. nutans* transcriptome (Finn et al., 2015). A full-length of gene ubiquitin ligases was selected for further analysis.

The protein sequence was analyzed using the SMART online software (http://smart.embl-heidelberg.de/) (Letunic et al., 2015). BLASTP web site was used to align multiple ubiquitin ligases sequences (Herrera-Galeano et al., 2014). The phylogenetic trees of ubiquitin ligases from *P. nutans* and other species were constructed using the Mega 6.0 program (Stecher et al., 2014).

2.3. RNA preparation and real-time polymerase chain reaction analysis

Total RNA was extracted from moss tissues or *Arabidopsis* seedlings using CTAB method and TRIzol reagent (Takara, Dalian, China) (Wang et al., 2017a). Single-strand cDNA was synthesized from 4 μg of total RNA with 5 × All-In-One RT MasterMix (abm, Canada). RT-qPCR was performed using the Bestar® SybrGreen qPCR Master Mix (DBI Bioscience, Shanghai, China). The amplification procedures were initiated with a pre-denaturing step at 94 °C for 2 min and followed by denaturing (94 °C for 20 s), annealing (57 °C for 30 s) and extension (72 °C for 20 s) steps for 40 cycles during the second stage, and a final stage of 65–95 °C to determine melting curves of the amplified products. The reference sequence was *Pntubulin*, *Pptubulin* or *AtACTIN2*. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All reactions were completed in triplicate. The relevant primer sequences for RT-qPCR assay are all presented in Table S1.

2.4. Subcellular localization of PnSAG1 proteins

The *PnSAG1* coding sequence was PCR amplified using the primer pair (Table S1) and inserted into a *pBI221:p35S:GFP* vector to generate the transgene *p35S:PnSAG1-GFP*. Then the plasmid was purified and introduced into 4 week-old wild-type *Arabidopsis* mesophyll protoplasts as described by Yoo et al. (2007), and incubated overnight in the dark at 22 °C. Meanwhile, the protoplasts were transformed with the *pBI221:p35S:GFP* control vector. GFP signal and chlorophyll autoflourescence were detected using a confocal laser scanning microscopy at excitation wavelengths of 488 nm and 647 nm, respectively.

2.5. In vitro PnSAG1 activity assay

The *PnSAG1*, *E1* (*AtUBA1*) and *E2* (*AtUBC8*) coding regions were cloned into the PET28a vector with two His tag in C-terminal and N-terminal. The fusion proteins were expressed in *Escherichia coli* BL21 (DE3) strain and purified by affinity chromatography using Ni Sepharose High Performance (GE Healthcare) following the manufacturers' instructions. Ubiquitination assays were carried out as previously described with some modification (Mark et al., 2016). Briefly, the reaction of 30 μL final volume contained 100 mM Tris-HCl, pH7.5, 25 mM $MgCl_2$, 2.5 mM dithiothreitol (DTT), 20 mM ATP, 1 μg ubiquitin (sigma), 1 μg E1 (AtUBA1-His), 1 μg E2 (AtUBC8-His) and 1 μg E3 (PnSAG1-His). The reaction mixture was incubated at 28 °C for 3 h and stopped by the addition of SDS sample buffer with boiling for 5 min. The sample was separated by 8% SDS-PAGE and subjected to Western blotting using the anti-ubiquitin antibody (New England BioLabs), and visualized by using an enhanced chemiluminescence detection system.

2.6. Generation of PnSAG1-overexpressing transgenic Physcomitrella patens and Arabidopsis plants

To generate the *PnSAG1*-overexpressing transgenic *P. patens* plants,

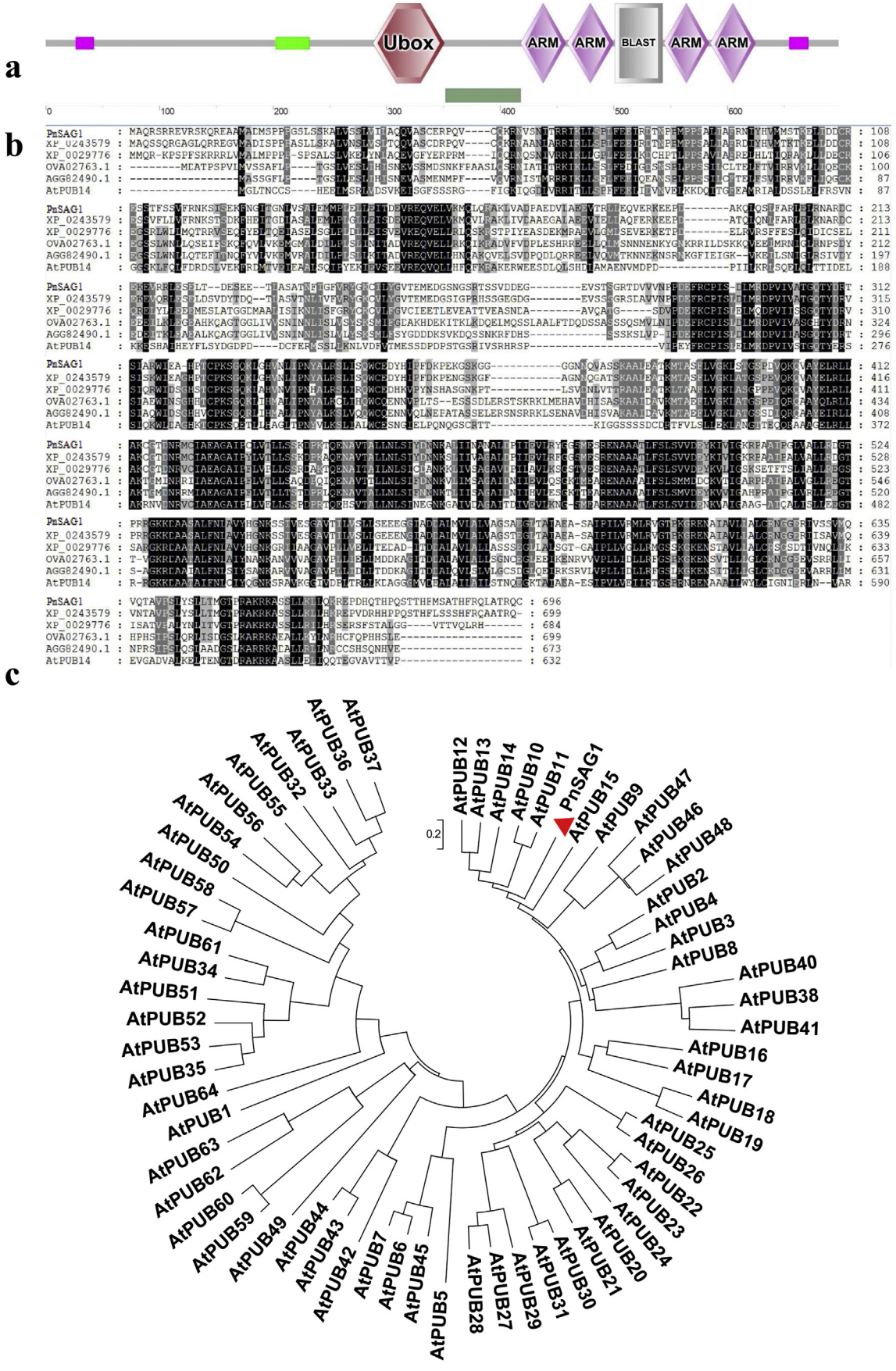

Fig. 1. Characterization and sequence analysis of PnSAG1.
a-b Amino acid sequence alignment of PnSAG1 with other U-box E3 ubiquitin ligases. **c** Phylogenetic relationship between PnSAG1 and other plant U-box proteins.

the *PnSAG1* sequence was inserted into the *pTFH15.3* vector according to the homologous recombination method as described by Zhang lab (Wang et al., 2017a). Before transformation, the *pTFH15.3:PnSAG1* plasmid DNA was linearised by digestion with restriction enzyme *Not* I and purified by standard phenol extraction/ethanol precipitation, and dissolved at a final concentration of 2 μg μL^{-1} sterile aqueous solution. 5d-old *P. patens* protoplasts were isolated following the modificated protocol of Cove et al. (2009). Then the plasmid was purified and introduced into *P. patens* protoplasts using polyethylene glycol (PEG)-mediated DNA transformation method with some key modification (Hohe et al., 2004). Briefly, 30 μg of linearised *pTFH15.3:PnSAG1* DNA fragments were mixed gently with 300 μL protoplast solution and 350 μL PEG solution (40% PEG 4000 and 0.3 M $Ca(NO_3)_2$in 3M medium, pH5.6). The mixture was incubated at room temperature for 30 min and carefully diluted with the gradient volume of 3M medium every 5 min. After centrifuged for 10 min, the transformed protoplasts was resuspended in 3 ml regeneration solution and incubated overnight in the dark at 25 °C followed by 8 days in the normal growth conditions. Afterwards, the suspension was spreaded on the cellophane covered BCD solid medium for 3 d and transferred to selection BCD solid medium containing antibiotic G418 (25, 50 or 100 μg mL^{-1}) to select for transformants. Moss surviving the third round of selection were regarded as stable transformants, and confirmed by PCR amplification of target fragments using specific gene primer pairs (Table S1).

To construct the transgenic *PnSAG1 Arabidopsis* plants, the full-length *PnSAG1* sequence was introduced into the modified binary *pROK2* vector. The constructs were transformed into *Arabidopsis* wild-type plants by *Agrobacterium*-mediated floral dip method (Zhang et al., 2006). Transgenic lines and their offspring were selected based on their resistance to kanamycin. Seedlings of T3 or T4 homozygous transgenic lines were subjected to phenotype and molecular analysis. We identified overexpressing lines by extracting genomic DNA and then confirmed the target fragments using PCR amplification.

2.7. Stress assays of transgenic Physcomitrella patens and Arabidopsis plants

For phenotype assays of transgenic *P. paten*, the same size stem tip of gametophytes of *PnSAG1* transgenic and WT plants were cut and grown on BCD agar medium containing different concentrations of NaCl and ABA. After cultured 30 d at 25 °C with 16 h light, the plants size was measured and the visual phenotypes were photographed. The experiments were replicated three times.

For phenotype assays of transgenic *Arabidopsis*, sterilized seeds were plated on 1/2 MS agar medium supplemented with various concentrations of NaCl and ABA, incubated in darkness at 4 °C for 2 d to break dormancy, and subsequently transferred to a 16 h photoperiod at 22 °C to allow germination. Germination rates were scored as the proportion of seeds that produced open green cotyledons. The seedlings roots length assay was conducted by sowing sterilized seeds on 1/2 MS agar medium containing different concentrations of NaCl and ABA. After growing for 7 d under stress conditions, the root growth was measured. The experiments were replicated three times.

2.8. Statistics analysis

The data of PnSAG1 transcriptional levels, seed germination, early root length and related genes expression were analyzed by a one-way ANOVA followed with Student's *t*-test. Error was presented as mean ± Standard Error (SE) and asterisks above columns in the figures indicate statistical differences (*$P < 0.05$).

3. Results

3.1. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignments indicated that PnSAG1 (Genbank accession number: MK905235) is homologous with proteins from other species, including *Physcomitrella patens* (XP_024357944.1), *Selaginella moellendorffii* (XP_002977628.1), *Macleaya cordata* (OVA02763.1), *Datisca glomerata* (AGG82490.1) and *Arabidopsis thaliana* (AtPUB14) (Fig. 1b). The amino acid sequence from PnSAG1 shared 84.6% identity with U-box type E3 of *P. patens*, whereas below 55.0% sequence identity with U-box type E3 of *S. moellendorffii*, *M. cordata*, *D. glomerata* and *Arabidopsis*. Analyses of the domain architectures revealed that PnSAG1 contained a U-box domain (amino acids 288–351) and four ARM domain (amino acids 418–625), which was first identified in *Drosophila Armadillo* and functioned in protein-protein interactions (Fig. 1a and b). These findings are consistent with the fact PnSAG1 is an U-box type E3. 64 U-box E3 proteins from higher plant *A. thaliana*, formed a phylogenetic tree to detect their evolutionary relationship (Fig. 1c). The results showed that PnSAG1 protein was closely related with AtPUB14 of *A. thaliana* (Fig. 1c).

3.2. PnSAG1 ubiquitin ligase activities and subcellular localization

The subcellular localization of PnSAG1 was investigated in *Arabidopsis* mesophyll protoplasts. In *p35S:GFP*-containing protoplasts, GFP fluorescence signal distributed evenly in the cell membrane, cytoplasm, and nucleus (Fig. 2a, v-viii). However, in *p35S:PnSAG1:GFP*-carrying protoplasts, PnSAG1:GFP signals were exhibited in cytoplasm, which is consistent with the predicted intracellular domain architecture analysis (Fig. 2a, i-iv). These results suggested that PnSAG1 might play a potential role in plant cytoplasm.

To test PnSAG1 ligase activity, we produced PnSAG1 in *E. coli* as a fusion protein with His-tag and affinity purified PnSAG1-His from the soluble fraction. In the presence of purified AtUBA1, AtUBC8, ubiquitin, ATP and reaction buffer, polyubiquitination activity was detected in the presence of the purified PnSAG1-His protein (Fig. 2b). However, in the absence of E1, E2, ubiquitin, ATP or reaction buffer, no polyubiquitination was observed. These results indicated that PnSAG1 had E3 ligase activity.

3.3. PnSAG1 expression induced by multiple abiotic stresses

To validate whether *PnSAG1* is involved in *P. nutans* response to abiotic stress, RT-qPCR assay was used to analyze the *PnSAG1* transcript patterns under multiple environmental stimuli. Under salt stress, the *PnSAG1* transcript induction reached a maximum peak with 4.1-fold at 0.5 h and maintained slightly higher induction with 1.7-fold at 3 h (Fig. 2c). Under cold stress, the *PnSAG1* expression levels peaked by 1.8-fold at 1 h, declined gradually after that and reached to basal level at 6 h (Fig. 2d). Simulated drought conditions led to abruptly highest transcript accumulation of *PnSAG1* at 2 h and remained high up at 6 h after stress (Fig. 2e). Under ABA treatment, *PnSAG1* expression was gradually upregulated from 0.5 h up to 1 h (Fig. 2f). Increased expression of *PnSAG1* by cold, salinity, drought and ABA revealed the stress-responsive character and ABA might mediate regulation of *PnSAG1* in *P. nutans*.

3.4. PnSAG1 reduced salt stress tolerance in transgenic Physcomitrella patens and Arabidopsis

To further illustrate the molecular functions of *PnSAG1*, we generated the transgenic *P. patens* and *Arabidopsis* plants constitutively overexpressing *PnSAG1*. Three independent transgenic *P. patens* (#1, #3 and #8) were confirmed by PCR analysis using specific primer pairs (Fig. 3c). To test the effect of *PnSAG1* on plant growth during salt stress,

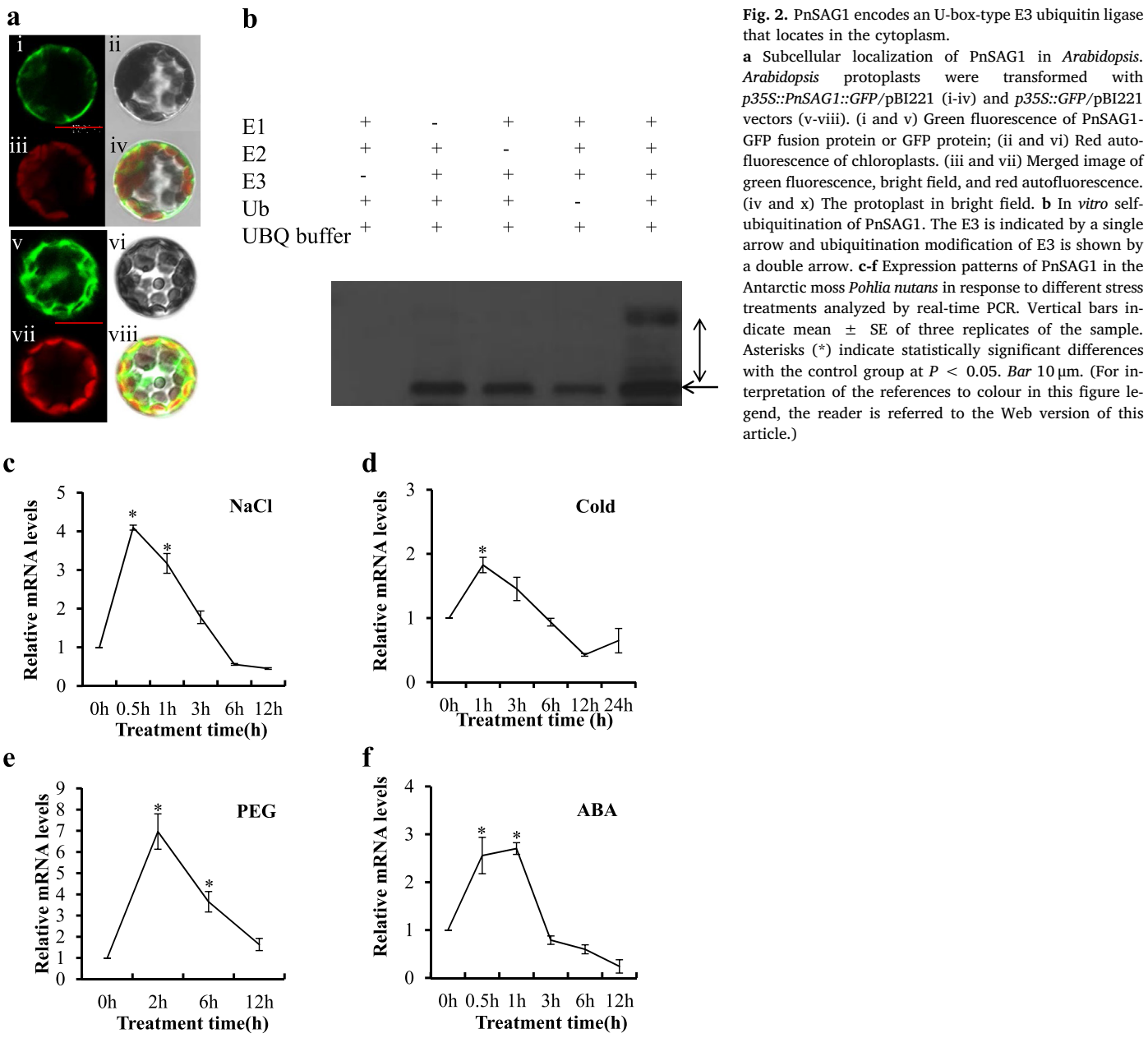

Fig. 2. PnSAG1 encodes an U-box-type E3 ubiquitin ligase that locates in the cytoplasm.
a Subcellular localization of PnSAG1 in *Arabidopsis*. *Arabidopsis* protoplasts were transformed with *p35S::PnSAG1::GFP*/pBI221 (i-iv) and *p35S::GFP*/pBI221 vectors (v-viii). (i and v) Green fluorescence of PnSAG1-GFP fusion protein or GFP protein; (ii and vi) Red autofluorescence of chloroplasts. (iii and vii) Merged image of green fluorescence, bright field, and red autofluorescence. (iv and x) The protoplast in bright field. **b** In *vitro* self-ubiquitination of PnSAG1. The E3 is indicated by a single arrow and ubiquitination modification of E3 is shown by a double arrow. **c-f** Expression patterns of PnSAG1 in the Antarctic moss *Pohlia nutans* in response to different stress treatments analyzed by real-time PCR. Vertical bars indicate mean ± SE of three replicates of the sample. Asterisks (*) indicate statistically significant differences with the control group at $P < 0.05$. *Bar* 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the same size stem tips of control and three transgenic lines were treated with varying concentrations of salt (0, 125 and 150 mM NaCl). Under normal conditions, control and transgenic lines showed equally well growth on BCD medium at 25 °C, and their seedling phenotypes were no obvious differences (Fig. 3a). However, the growth rates significantly decreased in the transgenic gametophytes with clone size of 5.8, 5.4 and 5.4 mm compared with 7.9 mm in control plants at 6 weeks on 125 mM NaCl medium. And the clone size of transgenic gametophytes were 4.4, 4.5 and 3.4 mm compared to 5.9 mm in control plants at 150 mM NaCl medium (Fig. 3b).

The *PnSAG1* transgenic *Arabidopsis* (#3 and #12) were confirmed by RT-qPCR with gene specific primers (Fig. 3h). All three lines showed 100% germination at 22 °C (control). However, following exposure to 125 or 150 mM NaCl, the germination rate of transgenic lines was markedly lower than that of WT plants (Fig. 3d). The germination rates of the transgenic plants decreased 43.4% and 68.7% at 125 mM NaCl, and 51.1% and 64.7% at 150 mM NaCl compared to WT plants, respectively (Fig. 3e). Moreover, in the presence of 125 mM NaCl, the primary root of WT plants was observed to form 2.0-fold longer than the transgenic plants, while there were no phenotypic difference between two transgenic plants and WT plants on standard medium (Fig. 3f and g).

3.5. PnSAG1 enhanced abscisic acid sensitivity in transgenic plants

It has been generally accepted that the plant tolerance to abiotic stresses may connected with ABA signaling pathway. As shown in Fig. 4a, the transgenic *P. patens* plants (#1, #3 and #8) showed smaller gametophytes size than the control plants after 5 or 10 μM ABA treatment. On 5 μM ABA, the gametophyte sizes of the transgenic *P. patens* plants were 6.0, 4.1 and 3.5 mm compared to 7.7 mm in control plants, while 3.5, 3.2 and 3.4 mm compared to 5.5 mm in control plants on 10 μM ABA (Fig. 4b).

In *Arabidopsis*, the germination rate analysis also exhibited that the transgenic plants were insensitive to ABA (Fig. 4c). In the presence of ABA, two transgenic seeds (#3 and #12) germinated slower than WT seeds. The germination rates of transgenic plants were 44.4% and 23.3%, while the germination rate of the WT plants was 70.0% on

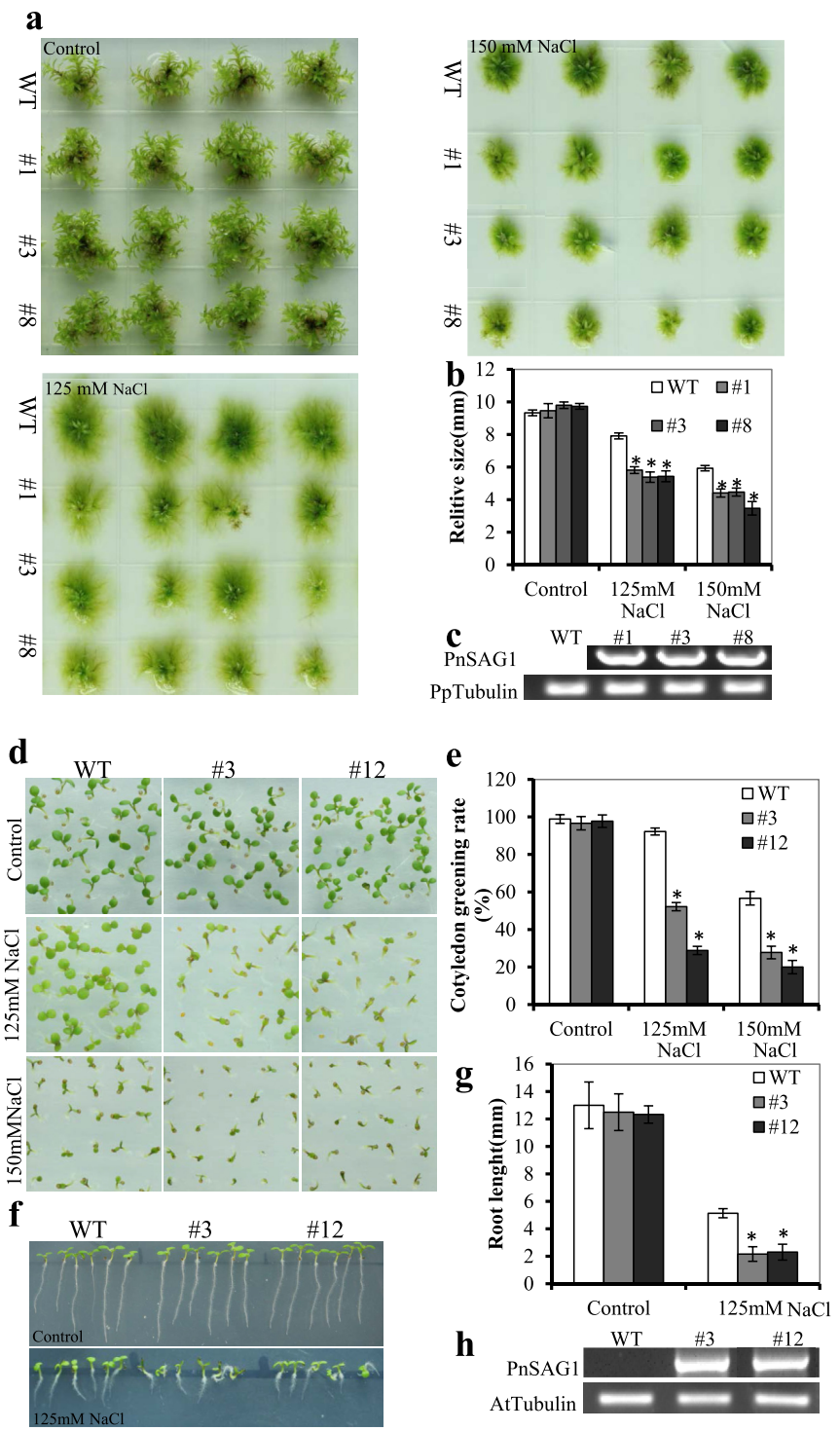

Fig. 3. PnSAG1 enhanced sensitivity to the salt stress in transgenic *Physcomitrella patens* and *Arabidopsis*. **a** The size of transgenic *Physcomitrella patens* gametophyte plants was significantly lower than that of the wild type under salt stress conditions (4-week-old plants). **b** Statistical analysis of gametophyte size as shown in **a**. **c** PCR analysis revealed that the *PnSAG1* was successfully transcribed in *Physcomitrella patens*. **d** The seed germination rate of transgenic seedlings was significantly lower than that of the wild type under salt stress conditions (6 days' seed germination). **e** Statistical analysis of seed germination rates as shown in **d**, seed germination rates were calculated by counting the proportion of the WT plants and the transgenic seedlings bearing open green cotyledons. **f** PnSAG1 inhibits the growth of *Arabidopsis* seedling after salinity treatment. **g** Measurement of root length of salinity-stressed *Arabidopsis* seedlings shown in **f**. **h** PCR analysis revealed that the *PnSAG1* was successfully transcribed in *Arabidopsis*. Vertical bars are presented as means ± SE, and asterisks (*) indicate significant differences of means between the transgenic lines and the WT plants at $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.5 μM ABA. Meanwhile, the germination rates of two transgenic plants were 17.8% and 12.2% compared with 28.9% of the WT plants on 0.75 μM ABA (Fig. 4d). For plants root length analysis, on ABA medium, the primary root growth of two transgenic plants were more impaired than that of WT plants. The root length of the transgenic plants decreased to 27.0% of the WT plants at 0.5 μM ABA (Fig. 4e and f).

3.6. *PnSAG1* reduced the expression of stress-responsive genes in transgenic *Physcomitrella patens* and *Arabidopsis*

To further determine the possible molecular mechanism of *PnSAG1* regulating salinity tolerance, the expression patterns of several stress-responsive genes (*PpABI3A*, *PpABI3B*, *PpABI3C*, *PpABI5A*, *PpABI5B*, *AtABI3*, *AtABI4*, *AtABI5*, *AtABF3*, *AtDREB2A*, *AtNCED3*, *AtRD22* and *AtRD29A*) was examined by RT-qPCR in the control and transgenic *P. patens* and *Arabidopsis* plants. These genes were involved in salt tolerance and ABA signaling pathway in *Arabidopsis*. After 2 h treatment with 200 mM NaCl, the transcript levels of PpABI3A, PpABI3B, PpABI3C, AtABI3, AtDREB2A, AtABF3 and AtRD29A showed significantly lower transcription accumulations in the transgenic plants than that of the control plants (Fig. 5a and b). However, the transcript levels of PpABI5A, PpABI5B, AtABI4, AtABI5, AtRD22 and AtNCED3 have no significant differences compared with WT plants (Fig. 5c and d). These results indicated that the increased salt sensitivity of the

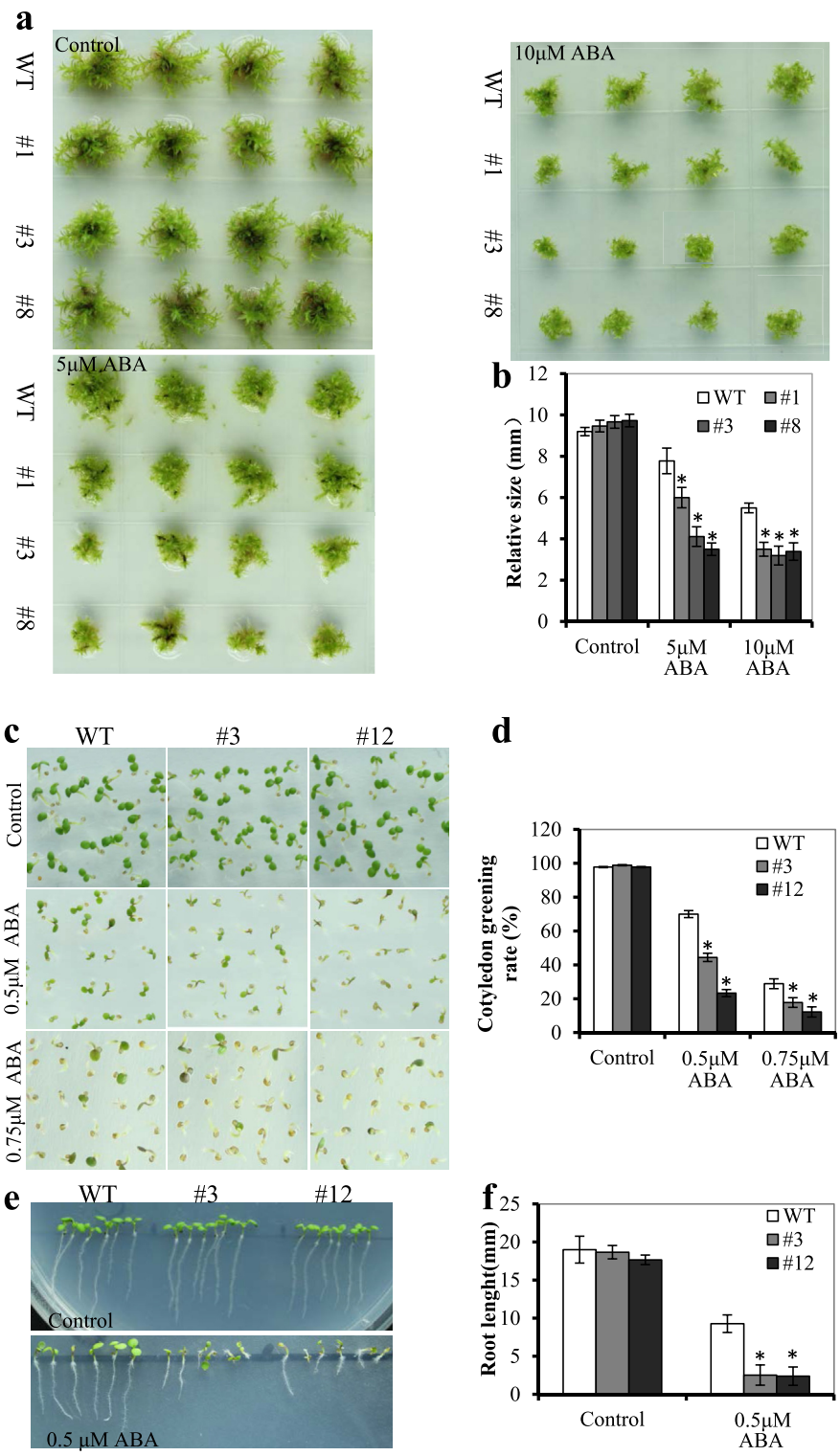

Fig. 4. PnSAG1 increased the ABA sensitivity in transgenic *Physcomitrella patens* and *Arabidopsis*.
a The size of transgenic *Physcomitrella patens* gametophyte plants was significantly lower than that of the wild type after ABA treatment (4 week-old plants). **b** Statistical analysis of gametophyte size as shown in **a**. **c** Seed germination of transgenic lines were significantly lower than that of the wild type under different concentrations of ABA (4 days' seed germination). **d** Statistical analysis of the transgenic *Arabidopsis* seedling greening shown in **c**. **e** PnSAG1 promotes the growth of *Arabidopsis* seedling after ABA treatment. **f** Statistical analysis of the root length in transgenic *Arabidopsis* shown in **e**. Vertical bars are means ± SE, and asterisks (*) indicate significant differences of means between the transgenic lines and the WT plants at $P < 0.05$.

PnSAG1 transgenic lines resulted from the lower expression of these stress responsive genes.

4. Discussion

The U-box type E3 ligases, which could interact with and degrade the specific substrates by ubiquitination, are involved in plant growth and development (Li et al., 2012; Kinoshita et al., 2015; Wang et al., 2017a,b), innate immunity (Liu et al., 2015) and abiotic stress (Trujillo, 2018). In this study, we identified a representative U-box armadillo repeat (PUB-ARM) ubiquitin ligases (PnSAG1) from *Pohlia nutans*, and it shares relatively high similarity with other PUB proteins including *Arabidopsis* and the rice (Fig. 1b). Previous studies have shown that the localization of PUB-ARM proteins was diverse, such as in the nucleus and the cytoplasm (BnARC1, TaPUB1, AtPUB30 and AtPUB46), in proteasome structures at the ER (BnARC1), in the cis-Golgi (GmPUB8), and in the plasma membrane (AtPUB30 and AtSAUL1) (Stone et al., 2003; Drechsel et al., 2011; Wang et al., 2016; Zhang et al., 2017a,b; Adler et al., 2018; Peng et al., 2019). Subcellular localization analysis showed that PnSAG1 located in the cytoplasm (Fig. 2a). It is notable

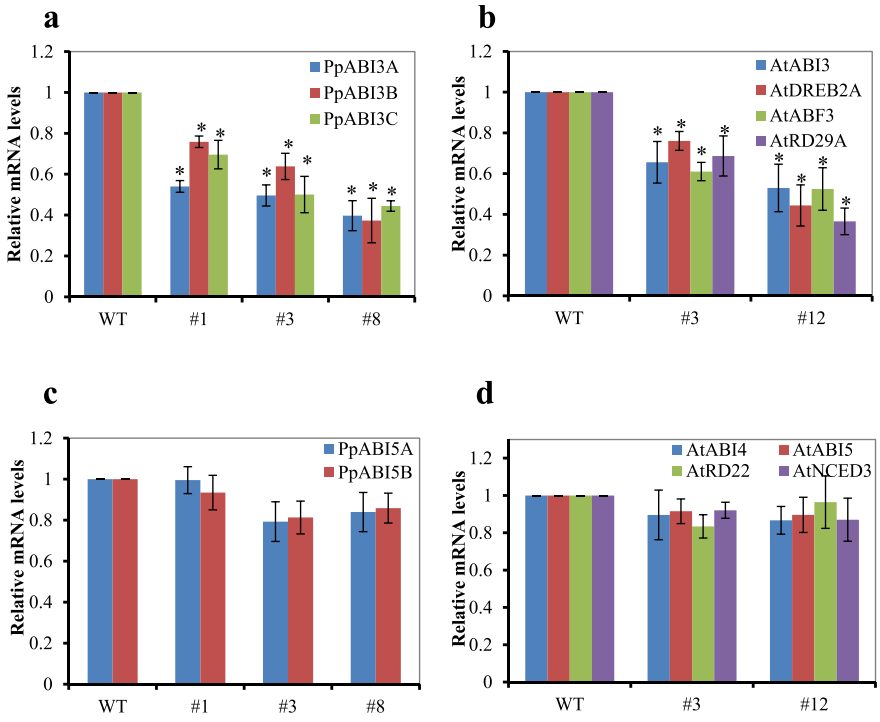

Fig. 5. The stress-responsive genes expression pattern in PnSAG1 transgenic *Physcomitrella patens* and *Arabidopsis*. The expression levels of several abiotic stress/ABA-related genes were measured by RT-qPCR. The *Physcomitrella patens* tubulin gene and *Arabidopsis* actin gene were served as normalization (Table S1). Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. The reactions were performed in triplicate.

that functioning as the largest subgroup of E3 ubiquitin ligases (more than 64%), PUB-ARM proteins capable of mediating polyubiquitination (Trujillo, 2018). Polyubiquitination was detected by anti-Ub antibody only when E1, E2, and PnSAG1 were all present. When any of them were omitted in the assay, polyubiquitination was not detected (Fig. 2b). It means that PnSAG1 possesses E3 Ub ligase activity in *vitro*. Furthermore, an inducible expression pattern of *PnSAG1* was observed under different treatments (ABA, NaCl, PEG and cold stress), which suggests the potential regulatory role of *PnSAG1* in plant response to abiotic stress (Fig. 2c–f).

A previous study revealed that PUB proteins possess biological significance under abiotic stress (Zhang et al., 2015). For example, pub12/pub13 or pub18/pub19 mutants plants were less sensitive to salt- and drought-dependent inhibition of germination in *Arabidopsis* (Bergler and Hoth, 2011; Kong et al., 2015). The overexpression of the soybean PUB8 in *Arabidopsis* hypersensitive to drought and NaCl during seed germination and seedling growth (Wang et al., 2016). *Arabidopsis* PUB30 negatively regulates salt tolerance probably through facilitating the degradation of BRI1 kinase inhibitor 1 (BKI1) and brassinosteroids signaling (Zhang et al., 2017c). In the present study, we also found that the PnSAG1-overexpressing *Arabidopsis* and *P. patens* both displayed hypersensitivity to salt stress during germination, post-germination growth and root growth (Fig. 3).

The phytohormone ABA has been regarded as a major plants endogenous messenger in controlling cellular and physiological responses to abiotic stresses (Raghavendra et al., 2010). Microarray expression profiles study has suggested that the ABA signaling pathway is conserved among land plants (from bryophytes to higher plants) (Richardt et al., 2010). Multiple ABA signaling transducers are subjected to the regulations by ubiquitination and proteolytic systems (Yu et al., 2016). Several reports have underlined the role of PUBs in ABA signaling process. Plants lacking SAUL1 were impaired in ABA-induced inhibition of germination, suggesting that they are less sensitive to ABA (Salt et al., 2011). In seedlings, PUB18 was the target of Exo70B1 for degradation and, accordingly, exo70B1 plants were less responsive to ABA (Seo et al., 2016). In rice, PUB70 mutants were less sensitive to ABA, while overexpression resulted in the opposite effect (Tang et al., 2016). Therefore, we further investigated the function of PnSAG1 in plant response to ABA. PnSAG1 overexpression in *P. patens* exhibited hypersensitivity to ABA in terms of gametophytes growth (Fig. 4a and b). Moveover, PnSAG1 overexpression *Arabidopsis* leads to increased sensitivity to ABA during the seed germination and early root growth stage (Fig. 4c–f). It has been suggested that PUB9 may act upstream of ABI3, whereas AtCHIP may positively regulate PP2A activity (Luo et al., 2006; Samuel et al., 2008). In this study, the transcript levels of stress-inducible genes including AtABI3, AtDREB2A, AtABF3, AtRD29A, PpABI3A, PpABI3B and PpABI3C displayed reduced induction in PnSAG1-overexpressing lines compared with WT plants following salt treatment (Fig. 5a and b). However, the transcript levels of PpABI5A, PpABI5B, AtABI4, AtABI5, AtRD22 and AtNCED3 have no significant differences compared with WT plants (Fig. 5c and d). These results further support that PnSAG1 negatively regulates salt stress responses. In conclusion, the data presented here provide the phenotypic and genetic evidence in support of a negative role of PnSAG1, a novel U-box domain protein, in ABA and salt stress signaling.

Author contribution statement

PZ, KC and SL conceived and designed research. JW and HL conducted experiments. JW and PZ analyzed data. JW, SL and PZ discussed the results. JW wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.002.

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